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Colony formation by human T lymphocytes in agar medium

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SUMMARY

An improved method is described for growing human T-lymphocyte colonies in agar medium containing phytohaemagglutinin (PHA) and sheep red blood cells (SRBC). Cluster and colony growth was obtained when blood mononuclear cells were plated directly in the agar-medium (*one-step* procedure) or after incubation of cells in liquid medium with PHA (*two-step* procedure). In the *one-step* procedure approximately 1 per 100 cells plated formed a cluster containing four to fifty cells. In the *two-step* procedure 1 per 20 cells plated formed a cluster or a colony (more than fifty cells). The proliferating cells were shown to be sheep-erythrocyte rosette-forming cells (E-RFC). Optimal proliferation was dependent on the presence of phagocytic cells in the cell suspensions cultured. No growth occurred in cultures depleted of E-RFC. Detailed studies of the cycle, velocity sedimentation, and density of the cells plated showed that the majority of cluster- and colony-forming cells were small non-cycling lymphocytes with a sedimentation velocity of 4 mm/hr, and a density between 1·069 and 1·077 g/cm^3.

INTRODUCTION

A recent development in the study of human lymphocytes has been the adaptation of a semi-solid agar culture procedure to permit the growth of T-lymphocyte colonies from peripheral blood mononuclear cells (Rozenszajn, Shoham & Kalechman, 1975; Fibach, Gerassi & Sachs, 1976; Wilson & Dalton, 1976). The number of human T-colony-forming cells was reported to be three to ten per 10,000 cells cultured, and growth of such cells required the continuous presence of phytohaemagglutinin (PHA), first in liquid culture for 18–24 hr, and then in agar. Also essential was a seeding level above 10^5 cells per ml of agar medium.

The present work described an improved method for growing T-lymphocyte colonies from human blood and examines several aspects of colony formation: (1) whether the reported low frequency of cells forming colonies reflects low circulating numbers of these cells or merely inadequacy of the culture conditions; (2) the lineage and nature of the mononuclear cell which generates T-lymphocyte colonies; (3) the necessity for PHA in liquid culture; (4) the dependence of plating efficiency on the number of cells cultured and (5) the effect of various cell-separation procedures on lymphocyte proliferation in agar cultures.

MATERIALS AND METHODS

Peripheral blood leucocytes. Blood from healthy adults was drawn into sterile flasks containing preservative-free heparin at a concentration of 10 iu per ml blood and two types of cell suspensions were prepared: (a) mononuclear cells were separated from whole blood by centrifugation through Isopaque–Ficoll of density 1·077 g/cm^3 (Böyum, 1968) and (b) leucocytes were separated from whole blood by sedimenting the erythrocytes through methyl cellulose-urografin (Dwyer & Mackay, 1970). The cell suspensions were washed twice and resuspended in Eisen's balanced salt solution containing 10% foetal calf serum (EBSS-F). Counts of viable cells were made using eosin-dye exclusion.

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Lymphocyte stimulation with PHA. One million leucocytes were suspended in 1 ml of Eagle's minimum essential medium (MEM) containing 10% foetal calf serum, and were incubated with 2 μl of PHA (Burroughs Wellcome Reagent Grade K1231) at 37°C in an atmosphere of air and 5% CO_2 for 18 hr. The PHA-treated cells were washed twice, resuspended and cell aggregates dispersed mechanically. Viable cell counts were then made.

Agar culture. In the *one-step* procedure 1×10^4 to 5×10^4 freshly separated viable mononuclear cells were plated. In the *two-step* procedure $2{\cdot}5\times10^3$ to 25×10^3 PHA pre-stimulated viable cells were plated. A volume of 0·2 ml of cells was added to 5 ml of equal parts of double-strength Dulbecco's modified Eagle's medium (DME) and 0·9% agar (Difco Bacto-agar) in double-distilled water previously boiled for 2 min, and held at 37°C. The composition of the double-strength DME has been described in detail elsewhere (Metcalf *et al.*, 1975). PHA and thrice washed sheep red blood cells (SRBC) were added to give a final concentration of 2 μl PHA and 1% erythrocytes per ml agar medium. 1 ml of the agar medium containing the cells was then pipetted into 35-mm plastic petri dishes and allowed to gel. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 in air for 5–7 days after which time the SRBC were lysed routinely by addition of 0·5 ml 3% acetic acid before examination in an Olympus dissection microscope. When the cells grown in agar were harvested (see below) SRBC were lysed by the addition of 0·5 ml 0·17 M NH_4Cl. Discrete groups of four to fifty cells were scored as clusters and groups of more than fifty cells were scored as colonies.

Harvesting of clusters and colonies. On day 5–7 of culture intact groups of proliferating cells were transferred with a fine Pasteur pipette to a drop of EBSS. The cells were dispersed with a 26-gauge needle and washed twice in siliconised Wasserman tubes.

Tests for cells bearing specific cell-surface receptors. E-rosette-forming cells (E-RFC) were tested for by the method of Kaplan & Clark (1974) using 2-aminoethylisothiouronium bromide (AET)-treated SRBC, and overnight incubation at 4°C. The pellet was gently dispersed after addition of acridine orange (Brostoff, 1974) and transferred to a microscope slide for examination by fluorescence microscopy.

Surface-membrane immunoglobulin (SMIg)-bearing cells were tested for by immunofluorescence using a fluorescein-conjugated Fab fragment of rabbit anti-human IgM. Cells were held with this reagent for 30 min at 4°C, washed twice and examined by fluorescence microscopy.

Fc-receptor-bearing cells, erythrocyte antibody RFC (EA-RFC), were tested for by rosette formation using SRBC treated with rabbit anti-SRBC haemolysin. SRBC were held with haemolysin (half minimum agglutinating dose) at 37°C for 30 min, washed thrice and adjusted to a 1% suspension. Equal volumes of haemolysin-treated erythrocytes and lymphocytes were mixed, centrifuged at 200 *g* for 2 min, held at room temperature for 30 min, resuspended after addition of acridine orange and examined by fluorescence microscopy.

Velocity sedimentation. Cells were layered above a buffered step gradient of from 7–30% foetal calf serum using an 11 cm glass sedimentation chamber (Johns Scientific, Toronto). Separation was carried out at unit gravity using the procedure of Miller & Phillips (1969). 30 to 50×10^6 cells were added and allowed to sediment for 3 hr at 4°C. Fractions of 10 ml were collected and the cells centrifuged at 400 *g* at 4°C. After resuspension in 0·5–1·0 ml phosphate-buffered saline (PBS) containing 10% foetal calf serum counts were made in a haemocytometer.

E-RFC enriched and depleted mononuclear cell suspensions. Mononuclear cell suspensions at concentrations of 2×10^6 cells/ml were mixed with equal volumes of 0·5% normal or AET-treated SRBC for 15 min at 37°C, centrifuged at 200 *g* held at 4°C for 2 hr, resuspended gently, and centrifuged through Isopaque–Ficoll for 30 mins at 200 *g*. Both non-E-RFC and E-RFC were recovered and washed in EBSS-F.

Density-cut separation. Mononuclear cells (10×10^6) were suspended in 1 ml of albumin of density 1·069 g/cm³, and layered over 1 ml of albumin of the same density. One ml of PBS was added above the albumin and the interfaces mixed. The cells were then centrifuged at 3500 *g* for 15 min. Cells of density lighter than 1·069 g/cm³ remained in suspension and denser cells formed a pellet. Supernatant and pelleted cells were harvested, separately washed, and counts of viable cells performed.

Depletion of phagocytic cells. Phagocytic cells were removed from mononuclear cell suspensions according to the method of Lundgren, Zukosi & Moller (1968). Briefly, 10×10^6 mononuclear cells were incubated at 37°C for 40 min in 10 ml MEM containing 10% FCS and 250 mg carbonyl iron followed by application of a magnetic field. The non-retained cells were washed twice and viable cell counts performed. The cell recovery was 70–80%.

In vitro suiciding. Selective destruction of cells in S phase was performed by incubating mononuclear cells with hydroxyurea (HU) (Sinclair, 1967). Briefly, 1×10^6 cells were incubated in 1 ml MEM containing 10% FCS for 1 hr at 37°C, with or without HU (2 mM/ml), then washed twice and cultured according to the *one-* and *two-step* procedure. S-phase blocking was confirmed in experiments with normal human bone-marrow cells incubated in MEM containing 10% FCS for 1 hr at 37°C with and without addition of HU. After incubation the cells were washed twice and cultured in agar-medium for development of granulocyte and macrophage colonies according to the method of Bradley & Metcalf (1966).

RESULTS

One-step procedure

No proliferation occurred when blood mononuclear cells were plated directly into the agar medium without PHA, with or without SRBC. Occasionally, clusters of macrophages were seen at day 7 in such cultures. When PHA and SRBC were present, clusters of 4 cells were seen at day 3. Despite the high concentration of agar (0·45%) proliferating cells were mobile in the agar forming clusters of loosely dispersed cells (Fig. 1a). When the concentration of agar was reduced to 0·3% proliferating cells dispersed freely within the agar obscuring the individual development of cell clusters.

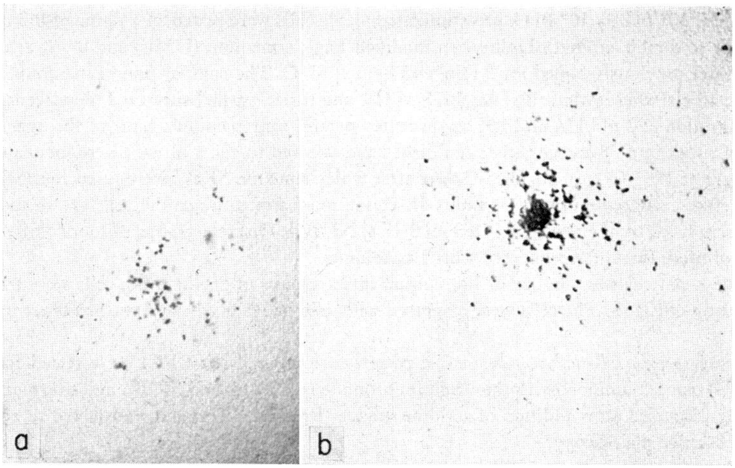

FIG. 1. A cluster and a colony cultured according to the *one-step* procedure (a) and the *two-step* procedure (b).

Cell numbers in proliferating groups. The number of cells in groups rarely exceeded 50 at day 7 because cell proliferation paralleled cell death probably due to the high concentration of agar used. This is illustrated in Table 1. Clusters from 5–7-day-old cultures were stained with aceto-orcein and counts of viable, pyknotic and dividing cells were made. The mean number of cells per cluster (29) remained constant over the 5–7-day period, although the mitotic index decreased ten-fold and the pyknotic index increased by a factor of five during this interval. Morphologically, the proliferating cells were large blast-like cells with a round, oval or sometimes spindle-shaped nuclei containing one or two prominent nucleoli.

Cell dose response. When cells were seeded in numbers varying from 2500 to 10,000 per ml medium, approximately 1 per 100 cultured cells formed a cluster (Fig. 2).

PHA dependence. The number of clusters was highly dependent on the concentration of PHA in the agar medium (Fig. 3). Optimal results were obtained with 2 μl of PHA per ml agar medium.

Starting cell population. No cell proliferation was observed when unfractionated blood leucocytes were cultured in agar medium. Preliminary removal of polymorphs was required before proliferation was obtained in the *one-step* procedure. On the other hand, partial removal of phagocytic cells from a pure mononuclear cell suspension reduced the number of clusters by approximately 65% (Table 2).

TABLE 1. Number of interphase cells, cells in mitosis and pyknotic cells in clusters (*one-step* procedure)

Days in culture	Cells per cluster*	Mitotic index (%)	Pyknotic index (%)
5	29 ± 15†	4·1	3·5
6	29 ± 13	1·1	9·1
7	29 ± 10	0·4	17·7

* Ten separate clusters were studied on 3 consecutive days.

† ± Standard deviation of the mean.

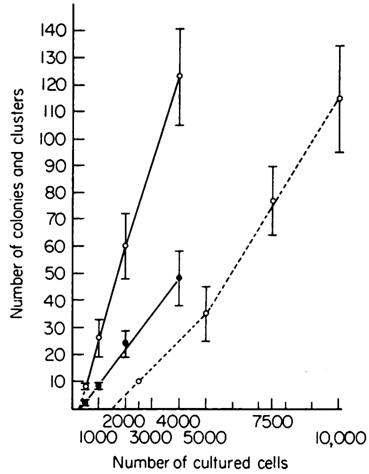

FIG. 2. Relationship between number of cultured cells and number of 7-day clusters (○) and colonies (●) in the *one-step* (- - -) and *two-step* (——) procedures. Vertical bars represent ±1 s.d. of the mean of four replicate cultures.

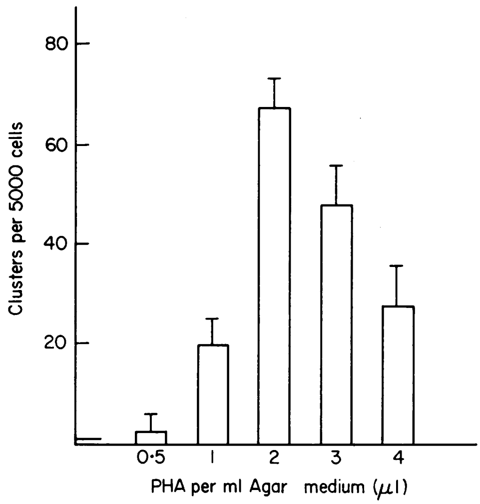

FIG. 3. Dependence of PHA in the agar medium (*one-step* procedure).

TABLE 2. Per cent reduction in numbers of clusters and colonies after partial removal of phagocytic cells

Experiment	*One-step** clusters (%)	*Two-step** Clusters (%)	Colonies (%)
I	65	66	64
II	68	100	100
III	47	84	87
IV	74	87	80
Mean	64	84	83

* Cultures contained 10,000 cells (*one-step*) or 2000–5000 cells (*two-step*) per ml agar medium.

Two-step procedure

Mononuclear blood cells were stimulated in liquid medium containing PHA for 18 hr, washed, and seeded into 0·45% agar medium containing PHA and SRBC. Both PHA and SRBC were essential for cell proliferation, the dependence on PHA being similar to that for the *one-step* procedure. Cells were seen to divide from the second day of culture and develop into clusters and colonies (more than fifty cells) at day 6–7 of culture. Again low-concentration agar medium (0·3%) allowed movement of newly formed cells to such a degree that their individual origin could not be established. A typical colony is shown in Fig. 1b. As in the *one-step* procedure, the cells stopped dividing from the 7th day of culture but cell numbers in the largest colonies reached about 200. At all stages of culture the morphology of the proliferating cells was identical in the two methods described.

Cell dose response. Fig. 2 shows the results of a typical dose–response experiment. Approximately 1 per 150 cultured cells formed a colony and nearly 1 per 20 cells formed a cluster. It is apparent (Fig. 2) that low numbers of plated cells did not give rise to cluster or colony formation indicating that cell division in agar medium may require, in addition to PHA and SRBC, some special cells or cell products which are diluted out when low cell numbers are cultured. Furthermore, a minimum number of cells (50,000/ml) was required during the period of incubation in liquid medium to obtain subsequent proliferation in agar medium of PHA-presensitized cells (Fig. 4).

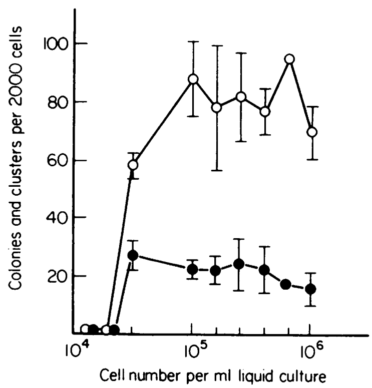

FIG. 4. The effects of cell numbers in the liquid step on numbers of clusters and colonies (*two-step* procedure). (●) Colonies; (○) colonies + clusters.

Starting population. In contrast to the *one-step* procedure, preliminary removal of polymorphonuclear leucocytes was not necessary to obtain cluster and colony formation. Partial removal of phagocytic cells from the mononuclear cell suspension prior to liquid culture on the other hand, reduced the number of clusters and colonies by approximately 80% (Table 2).

Nature of proliferating cells. The frequencies of E-RFC, EA-RFC and SMIg-bearing cells in mass-harvested cluster and colony-cell suspensions are shown in Table 3. The Table includes data from freshly prepared mononuclear-cell suspensions, cells incubated in liquid medium with PHA and cells cultured for 1 hr in agar medium. The presence of agar markedly influenced the number of cells with SRBC- and IgM-receptors, whereas counts of EA-RFC were not influenced by seeding in agar medium. Considering these effects of agar on lymphocyte receptors the data in Table 3 indicate that at least 30% of the cluster cells in the *one-step* procedure and about 80% of the cluster and colony cells in the *two-step* procedure are E-RFC. The high frequency of EA-RFC in cell suspensions incubated with PHA together with the absence of Ig-bearing cells in these preparations suggests that the cells proliferating in the agar medium are of the T-lymphocyte lineage.

Separation of cluster- and colony-forming cells. Human mononuclear blood cells were fractionated using

TABLE 3. Surface markers on cells harvested from clusters and colonies*

Cells	E-RFC (%)	EA-RFC (%)	SMIg bearing (%)
Mononuclear cells (MC)	60–75	30–45	5–10
One-step	20–30	10–40	n.t.
Two-step	40–80	30–36	0
MC/1 hr in agar	45	30	n.t.
MC/24 hr with PHA	85–95	60–70	<1

n.t. = Not tested.

* Data are from two to four experiments

velocity-sedimentation separation. The individual fractions were cultured according to the *one-* or *two-step* procedure and the sedimentation velocity of the cells generating clusters and colonies as well as the sedimentation profile of E-RFC were determined. Five separate experiments were carried out and the results were highly reproducible. Typical results are shown in Figs 5–7. The majority of cells forming clusters and colonies segregated as a single peak, which coincided with the small lymphocyte peak showing a sedimentation velocity of approximately 4 mm/hr. As indicated in Figs 5 and 6 some rapidly sedimenting cells (large cells) were also capable of proliferation. The sedimentation velocity of E-RFC and of all mononuclear cells is shown in Fig. 7. The majority of E-RFC segregated as a single peak coincident with that of cluster- and colony-forming cells.

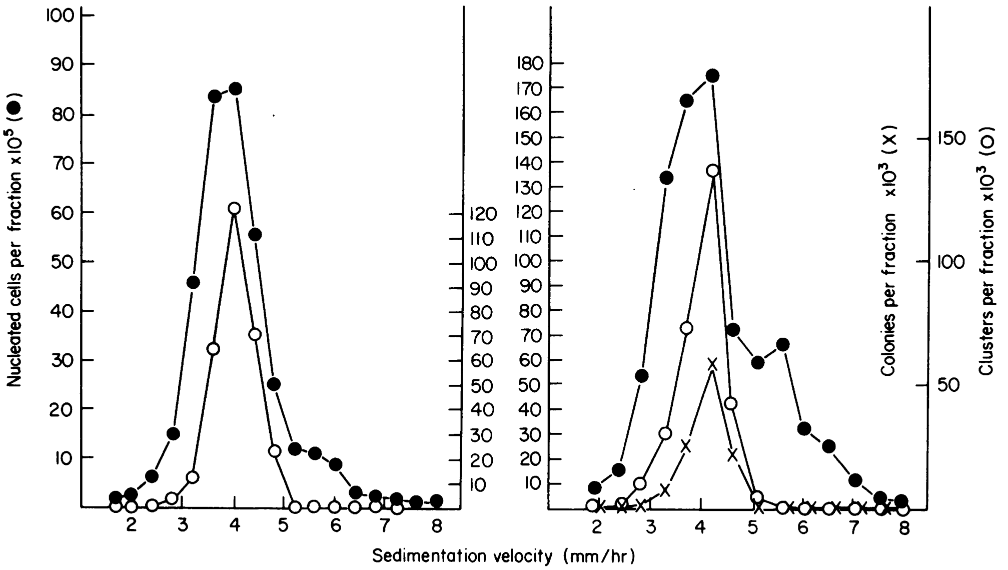

FIG. 5 FIG. 6

FIGS 5–6. Velocity-sedimentation separation of mononuclear blood cells showing fractions of cells forming clusters and colonies according to the *one-step* procedure (Fig. 5) and *two-step* procedure (Fig. 6). Mean colony counts from four replicate cultures.

When mononuclear cells were separated according to density it was found that about 90% of all cluster- and colony-forming cells exhibited a density higher than 1·069 g/cm^3. Thus, 90% of the cells forming clones in agar have densities between 1·070 and 1·077 g/cm^3 (density of Isopaque–Ficoll) which corresponds with the density profile of small lymphocytes.

Depletion of E-RFC. Cell suspensions were depleted of E-RFC by separation on an Isopaque–Ficoll gradient (see the Materials and Methods section). Three types of experiment were performed: (1)

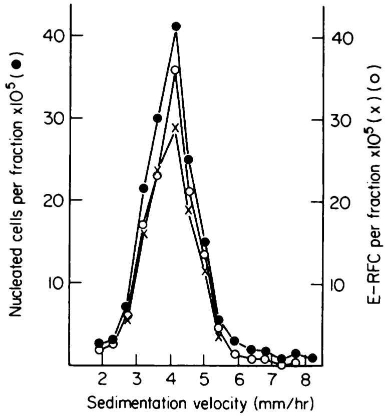

FIG. 7. Velocity-sedimentation separation of mononuclear blood cells showing localization of E-RFC. (×) E-RFC prior to liquid incubation with PHA. (○) E-RFC after 18 hr in liquid culture with PHA.

TABLE 4. Cluster and colony growth from E-RFC-depleted and mononuclear cell (MC) suspensions

*One-step**		*Two-step**					
		MC		E-RFC-depleted†		E-RFC-depleted‡	
MC clusters	E-RFC-depleted clusters	Clusters	Colonies	Clusters	Colonies	Clusters	Colonies
340±53	6±3	75±20	16±7	2±2	0	7±2	0

Data represent mean colony and/or cluster counts per 10,000 cells (*one-step*) and 2,000 cells (*two-step*) ± s.d. from four replicate cultures.

* Cultures contained 10,000 cells (*one-step*) or 2000–5000 cells (*two-step*) per ml agar medium.
† E-RFC-depletion prior to PHA-liquid culture.
‡ E-RFC-depletion after PHA-liquid culture.

TABLE 5. Influence of 1 hr hydroxyurea (HU) treatment on growth of T-lymphocyte clusters and colonies* and bone-marrow GM-CFC†. Data represent mean cluster or colony counts ± s.d. from four replicate cultures.

Experiment	*One-step* clusters		*Two-step* Clusters		*Two-step* Colonies		Bone-marrow cells GM-CFC‡	
	GM-CFC	HU	GM-CFC	HU	GM-CFC	HU	GM-CFC	HU
I	277±12	216±38	50±22	27±3	22±3	15±5	195±65	112±24
II	221±30	267±6	53±20	88±48	18±3	36±6	180±33	105±25
III	n.t.	n.t.	42±8	52±4	24±3	23±4	123±19	93±13
IV	n.t.	n.t.	39±3	47±3	23±4	19±5	n.t.	n.t.
V	n.t.	n.t.	45±11	57±3	25±3	21±3	n.t.	n.t.
Mean	234±13§	241±26	46±3	54±10	22±1	23±4	166±17	103±4

n.t. = Not tested.

* Cultures contained 10,000 cells (*one-step*) or 2000 cells (*two-step*) per ml agar medium.
† Cultures contained 100,000 cells per ml agar medium. Stimulus was a monkey lung-conditioned medium.
‡ Colonies and clusters.
§ s.e.m.

E-RFC-depleted cell suspensions were cultured according to the *one-step* procedure; (2) E-RFC-depleted cells were incubated in PHA-containing liquid medium and then plated according to the *two-step* procedure; and (3) cell suspensions were depleted of E-RFC after incubation with PHA and then plated in agar. In all these situations cell proliferation was minimal in the agar cultures (Table 4). Thus both the cells which respond to PHA in agar medium by proliferation and the cells which are sensitive to PHA in liquid medium and after seeding in agar develop into clusters and colonies seem to be E-RFC, i.e. T lymphocytes.

Suiciding experiments. Treatment of mononuclear cells with HU prior to culture did not influence cluster and colony formation (Table 5) indicating that the vast majority of cells activated by PHA are non-cycling lymphocytes. In contrast, HU-treated normal bone-marrow cells showed a reduction by 38% in their capacity to form granulocyte and macrophage clusters and colonies in agar medium—a reduction comparable to that obtained by tritiated-thymidine suiciding (Moore & Williams, 1973).

DISCUSSION

The present work shows that direct culture of freshly prepared human mononuclear blood cells (*one-step* procedure) as well as seeding PHA-presensitized cells into agar medium containing PHA and SRBC (*two-step* procedure) permitted some of the cultured cells to proliferate and form clusters and colonies composed of mononuclear cells which displayed the receptor for E rosettes. The frequency was found to be one proliferating cell per 100 directly plated cells and one proliferating cell per twenty PHA-presensitized cells. The majority of the cluster- and colony-forming cells had a density higher than 1·069 g/cm^3, were non-cycling and had a sedimentation velocity of 4 mm/hr similar to the value for the majority of E-RFC and small lymphocytes. Moreover, E-RFC-depletion experiments indicated that cluster-and colony-forming cells are E-RFC themselves, suggesting that they belonged to the T-lymphocyte lineage. The high frequency of Fc-receptor-bearing cells both after liquid incubation with PHA and in suspensions prepared from mass-harvested clusters and colonies is in line with recent work on activated T lymphocytes showing that more than 50% of human peripheral blood T cells will bind Ig after overnight incubation (Moretta *et al.*, 1975).

Peters (1972, 1974) reported that the degree of lymphocyte stimulation by PHA in liquid medium and in semi-solid agar medium is strongly dependent on cell crowding and that stimulation occurs exclusively within cell agglutinates induced by the lectin. Since the present *one-step* procedure completely excludes direct cell-to-cell contact we may conclude that single lymphocytes can be stimulated to mitotic activity by PHA. On the other hand a certain degree of cell crowding seemed to be important for lymphocyte activation because with low cell numbers, whether in the liquid PHA-presensitization step or in the culture dishes, there was no cell proliferation.

The findings of Lewis & Robbins (1970) showing strong dependency on macrophages of human lymphocytes activated by PHA, have been convincingly confirmed in guinea-pigs (Rosenstreich & Wilton, 1975). Macrophage-dependency of cluster- and colony-formation was evident in the present work where reduction in the number of cultured phagocytic cells by the carbonyl-iron method (Lundgren *et al.*, 1968) reduced the number of clusters and colonies by 60–80%.

In three recent papers on colony-formation in agar of human lymphocytes (Rozenszajn *et al.*, 1975; Fibach *et al.*, 1976; Wilson & Dalton, 1976) stimulation in liquid medium with PHA proved to be necessary for subsequent proliferation of cells in agar medium containing PHA. In these studies no SRBC were included in the medium and the frequency of colony-forming cells was found to be one per 1000–3000 plated cells. Moreover, no growth was demonstrated when cultured cell numbers were less than 10^5 per ml agar medium. The 10–100 times higher plating efficiency of the present *two-step* procedure, the relatively high efficiency of the *one-step* procedure and the loose, dispersed feature of growing clusters and colonies, suggesting extensive mobility of newly formed cells, could indicate that the lower figures of earlier reports reflect inadequate culture conditions. In the previous studies, many of the colonies were reported to be very large, appearing as 'tightly packed spheres of cells' (Wilson & Dalton, 1976) with up to 1000 cells in a single colony. In light of the present data on colony size and morphology, it seems possible that such large colonies might have been formed from small PHA-induced cell agglutinates developed during the liquid preincubation step, rather than from single cells. This could be one explanation of the relatively low cell-plating efficiency reported.

The high agar concentration (0·45%), used in the present studies affected the viability of proliferating cells but allowed more accurate counting. When the agar concentration was reduced to 0·4% both clusters and colonies contained higher numbers of viable cells but the increased cell mobility made counting difficult.

The simplicity of the present culture system for human peripheral blood T lymphocytes, the high frequency of proliferating cells, the requirement of only low cell numbers, and the demonstration that presensitization in liquid medium by PHA can be omitted should permit a wide range of studies on factors which influence human T-lymphocyte proliferation and function.

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REFERENCES

BÖYUM, A. (1968) Separation of lymphocytes from blood and bone marrow. *Scand. J. clin. lab. Invest.* 21, 51, supplement, 97.

BRADLEY, T.R. & METCALF, D. (1966) The growth of mouse bone marrow cells *in vitro*. *Aust. J. exp. Biol. med. Sci.* 44, 287.

BROSTOFF, J. (1974) A simple technique for counting rosettes using acridine orange. *J. Immunol. Methods* 5, 303.

DWYER, J.M. & MACKAY, I.R. (1970) Antigen-binding lymphocytes in human blood. *Lancet*, i, 164.

FIBACH, E., GERASSI, E. & SACHS, L. (1976) Induction of colony formation *in vitro* by human lymphocytes. *Nature (Lond.)*, 259, 127.

KAPLAN, M.E. & CLARK, C. (1974) An improved rosetting assay for detection of human T lymphocytes. *J. Immunol. Methods*, 5, 131.

LEWIS, W.R. & ROBBINS, J.H. (1970) Effect of glass adherent cells on the blastogenic response of 'purified' lymphocytes to phytohemagglutinin. *Exp. cell. Res.* 61, 153.

LUNDGREN, G., ZAKORI, C.H.F. & MOLLER, G. (1968) Differential effects of human granulocytes and lymphocytes on human fibroblasts *in vitro*. *Clin. exp. Immunol.* 3, 817.

METCALF, D., NOSSAL, G.J.V., WARNER, N.L., MILLER, J.F.A.P., MANDEL, T., LAYTON, J.E. & GUTMAN, G.A. (1975) Growth of B-lymphocyte colonies *in vitro*. *J. exp. Med.* 142, 1534.

MILLER, R.G. & PHILLIPS, R.A. (1969) Separation of cells by velocity sedimentation. *J. cell Physiol.* 73, 191.

MOORE, M.A.S. & WILLIAMS, N. (1973) Analysis of proliferation and differentiation of foetal granulocyte-macrophage progenitor cells in haemopoietic tissue. *Cell Tissue Kinet.* 6, 461.

MORETTA, L., FERRARINI, M., DURANTE, M.L. & MINGARI, M.C. (1975) Expression of a receptor for IgM by human T cells *in vitro*. *Europ. J. Immunol.* 5, 565.

PETERS, J.H. (1972) Contact co-operation in stimulated lymphocytes. I. Influence of cell contact on unspecifically stimulated lymphocytes. *Exp. cell. Res.* 74, 179.

PETERS, J.H. (1974) On the hypothesis of cell contact mediated lymphocyte stimulation. *Proc. Eighth Leucocyte Culture Conf. 'Lymphocyte recognition and effector mechanisms'* (eds K. Lindahl-Kiessling and D. Osoba), p. 13. Academic Press, New York and London.

ROSENSTREICH, D.L. & WILTON, J.M. (1975) The mechanism of action of macrophages in the activation of T lymphocytes *in vitro* by antigens and mitogens. *Proc. Ninth Leucocite Cult. Conf. Immune Recognition* (ed. by A.S. Rosenthal), p. 113. Academic Press, New York, San Francisco and London.

ROZENSZAJN, L.A., SHOHAM, D. & KALECHMAN, I. (1975) Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. *Immunology*, 29, 1041.

SINCLAIR, W.K. (1967) Hydroxyurea: effects on Chinese hamster cells grown in culture. *Cancer Res.* 27, 297.

WILSON, J.D. & DALTON, G. (1976) Human T lymphocyte colonies in agar: A comparison with other T cell assays in health subjects and cancer patients. *Aust. J. exp. Biol. Med. Sci.* 54, 27.